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Identification of ß-Catenin Gene as a Colorectal Cancer Controller in Mice *(Mus musculus)* Induced by Azoxymethane (AOM) and Dextran Sulfate Sodium (DSS) Using PCR RFLP Method with EcoR1 and Hinif1

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Abstract. Colorectal cancer is cancer attacking the colon to the rectum. The pathophysiology of colorectal cancer occurs due to several causes, such as changes in normal colonic epithelial cells histopathologically through molecular processes. Another cause is that the adenomatous polyps become colorectal cancer due to the carcinogenesis process. Most colorectal cancers originate from adenocarcinomas. Colon cancer is characterized by the uncontrolled growth of cells in the epithelial lining of the large intestine. This study aims to determine the mutation of the catenin gene in mice mus muculus induced by Azoxymethane (AOM) and Dextran sulfate sodium (DSS) using restriction enzymes EcoR1 and Hinif1. The type of this study was laboratory experimental research. The population was 2 mice that had been induced by AOM and DSS and 1 mouse did not get any treatment for 2 months. From the result, the results of RFLP on PCR products from 3 samples that had been used showed that only one sample showing the presence of the β-catenin gene by marking the formation of a 227bp Deoxy Nucleic Acid (DNA) band and testing the EcoR1 restriction enzyme did not show any cutting of DNA fragments with no DNA bands. The size of 81bp and 146bp for Hinf1 restriction showed a mutation in P3 with the formation of bands of 89bp and 138bp in the large intestine that had been induced by azoxymethane and dextran sodium sulfate. The implication of this research is that mutations in the β-catenin gene which are markers of colorectal cancer can be identified or can only be detected using the Hinif1 enzyme with the RFLP PCR method.

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Introduction

Cancer is a disease that occurs due to cell abnormalities caused by DNA mutation. The abnormal cell forms a clone and proliferates abnormally [1]. In cancer, there is an uncontrolled cell proliferation and a loss of capability in activating the apoptosis program causing cancer cells to be immortal [2]. Colorectal cancer is cancer attacking from the colon to the rectum [3].The pathophysiology of colorectal cancer happens due to several factors, such as changes in normal colonic epithelial cells histopathologically through a molecular event. Another factor is that adenomatous polyps become colorectal cancer due to a carcinogenesis process. Most colorectal cancers originate from adenocarcinomas [4]. The development of colorectal cancer is a step-by-step process starting from hyperplasia of mucosal cells, and then, adenoma formation and it develops from dysplasia to malignant transformation and invasive cancer [5][6].

AOM (Azoxymethane) is a colonic genotoxic carcinogen widely used for an investigation of the pathogenesis and carcinogenesis of colon cancers in Rodentia [7]. Meanwhile, DSS (Dextran sulfate sodium) is a synthetic sulfated polysaccharide that can be non-genotoxic colon carcinogens that are often used for causing inflammation of the colon (colitis) in Rodentia as a model against ulcerative colitis in humans [8]. DSS (Dextran Sodium Sulfate) is a collitogen material with anticoagulant properties, which is used to induce epithelial damage which is widely used to induce epithelial damage[9]. The study [10] examined mutations in the β-catenin gene in mice induced by AOM using the PCR-single strand conformation Polymorphism (SSCP) method, using restriction enzymes. In this study, experimental animals were only given the carcinogenic compound AOM, but DSS was not given as a step in the occurrence of inflammation in the colon. So it is not possible to know the stages of the mutation of the β-catenin gene, whether it occurs when the mice still have colitis or when colorectal cancer has occurred.

In Kohno's study, 2005, DMH/DSS-induced adenocarcinoma mice, using the PCR-SSCP method, detected the β-catenin gene using histological methods and immunohistochemical expression [11], but not using the Fragment Length Polymorphism (RFLP) method. RFLP is a technique using restriction enzymes to cut homologous DNA fragments to result in DNA fragments in different sizes from one allele to another allele; accordingly, it will demonstrate its homology [5]. This method is performed by using restriction enzymes to cut DNA molecules in its recognition sites. The sequence of restriction enzyme recognition is various, such as GAATTC for EcoR1, GGATCC for BamH1, GAGCTC for Sacl, and GANTC for Hinif1. Most restriction enzymes are palindromic which means that the recognition sequence is the same when reading 5’ to 3’ from both upper strand and lower strand [12].

Colon cancers are mostly initiated by activation of the abnormal β-catenin pathway. β-catenin, a cytoplasmic phosphoprotein through the Wnt signalling pathway, will be translocated to the nucleus. In the nucleus, β-catenin will interact with other transcription regulator for proliferation, cell division, cell adhesion, and embryogenesis up to mutation that can lead to carcinogenesis [13][14]. β-catenin is a nuclear-to-nuclear translocated gene that functions as a transcriptional activator with the binding of the DNA protein Tcf/Lef (Lymphoid enhancer factor/Tcell factor). The most important gene targets of this pathway are c-myc, Cyclin D1, c-jun, vascular endothelial growth factor (VEGF), matrix metalloproteinase-7 (MMP-7) and CD44 [12].

This study aims to identify the β-catenin gene encoding colorectal cancer in mice (Mus musculus) induced by azoxymethane (AOM) and Dextran Sulfate Sodium (DSS) using PCR RFLP (Restriction Fragment Length Polymorphism) method with enzyme EcoR1 and Hinf1. Nonetheless, not many studies that conducted an investigation on the application of the RFLP (restriction fragment length polymorphism) method in detecting β- catenin genes encoding colon cancer; hence, the researcher was interested in conducting research on the identification of β-catenin genes encoding colorectal cancer in mice (*Mus musculus*) induced by azoxymethane (AOM) and Dextran Sulfate Sodium (DSS) using the RFLP (Restriction Fragment Length Polymorphism) with EcoR1 and Hinf1.

Method

This study used two 8-week old mice (*Mus musculus*). The ethical clearance of this study was obtained from the Faculty of Medicine, *Universitas Hasanuddin* with the following registration number: UH20110627
The first injection was carried out by injecting a dosage (0.25 mL) of AOM that had been diluted to 100 mL of 0.9% NaCl through a peritoneal route. Giving DSS was done ad libitum for 5 days, a week after AOM injection and 1 mouse did not get any treatment.

Sampling. Mice (Mus musculus) were taken from the cage, and then, they were put into a container containing cotton and ether. Next, they were operated after being left until collapsed and their colons were taken out using a tweezer and stored in distilled water with pH 7. The sample was stored in a thermos or fridge until it would be used further [15].

DNA Extraction

Sample Preparation. The fresh colon sample was weighed of 25 mg and ground until it was well-minced and put into a 1.5ml microtube. Each GST Buffer of 200µl and Proteinase K of 20µl was added into the minced colon sample. The suspension was made homogenous using a vortex, and it was incubated at 60° C overnight (6 hours).

Cell Lysis stage. The sample that was not separated from its supernatant was centrifuged at a speed of 14,000g for 2 minutes. The supernatant was separated from its residue and put into a 1.5ml microtube. Into the 1.5ml microtube, 200µl of GSB buffer was added and the suspension was made homogenous for 10 seconds.

DNA Binding Stage. 200µl of absolute ethanol was added into the suspension and it was made homogenous for 10 seconds. The residue that had been formed was resuspended using a micropipette. GS Column was placed on the 2ml Collection tube. All collected suspensions (including insoluble sample residue) were moved into the GS Column and centrifuged at 14,000 - 16,000g for1 minute. If, after being centrifuged, the solution was not flowing through the GS Column membrane, so, during the centrifugation, it was improved until the entire solution passed through the membrane. The supernatant in the 2ml Collection Tube was discarded and the GS Column was moved into a new 2ml Collection Tube.

Cleaning the DNA. 400µl of W1 Buffer solution was added into the GS Column. The suspension was centrifuged at a speed of 16,000g for 30 seconds, and then, the supernatant was discarded. The GS Column was put into the Collection tube again. 600µl of Wash Buffer was added (make sure if absolute ethanol has been added) into GS Column and centrifuged at a speed of 16,000g for 30 seconds, and then, the supernatant was discarded. The GS Column was put into the Collection tube again and recentrifuged for 3 minutes at the same speed to dry up the matrix column.

Elution. The dried GS Column was moved into a 1.5 ml microcentrifuge tube. 100µl of Elution Buffer that was heated before was put into the GS Column. The suspension was left for 3 minutes to ensure if the Elution Buffer, TE Buffer, or water is fully absorbed. The suspension was then centrifuged at a speed of 14,000 - 16,000g for 30 seconds to gain pure DNA [16].

Measurement of DNA Concentration. The measurement of DNA concentration was carried out using UV-Vis spectrophotometry at a wavelength of 260nm, , which was the DNA absorption area. The DNA sample was put into a cuvette, Solution with an absorbance value of 1.0, proportionate to 50µg double-stranded DNA per ml and adjusted to Dilution Factor (DF) and then, it was put into the spectrophotometer. The measurement result of DNA concentration would appear on the monitor

of a spectrophotometer. The formula for measuring DNA concentration is as follows (DNA) = A260 x 50 μg/ml x DF [17]

DNA Amplification. For amplifying the DNA from β-catenin genes, a couple of primers, namely 5'- primer (GCTGACCTGATGGAGTTGGA) and 3'- primer (GCTACTTGCTCTTGCGTGAA) [10]. PCR was conducted by using My Taq HS in the reacting volume of 25μL consisting of 12.5μL of master mix, 0.5μL of each primer, 0.5μL of $MgCl_2$, 6μL of Water Free Nuclease, and 5μL of DNA template. The amplification process was performed in the PCR machine using pre-denaturation condition at 94ºC for 5 minutes, denaturation at 94ºC for 30 seconds, annealing at 57˚C for 1 minute, and extension at 72ºC for 1 minute 30 seconds, and final extension at 72˚C for 7 minutes. The PCR cycle was conducted 35 times. The PCR product was then analyzed using electrophoresis.

RFLP Analysis (Restriction Digestion). Fragment as the product of direct PCR amplification was used in the digestion reaction by using restriction enzymes. The amplification result using PCR was digested using EcoRI digestion consisting of 10μL of DNA (the result of DNA amplification), 1μL of EcoRI restriction enzyme, 1μL of Hinif1 restriction enzyme, and 17μL of nuclear-free water was added into each of them and put into a sterile microcentrifuge. Then, the mixture was centrifuged for 30 seconds at a speed of 14,000-16,000 rpm and incubated at 37˚C for 20 minutes [18]

The Analysis of PCR Products using Electrophoresis. To know the DNA amplification result, electrophoresis was applied to PCR products in 2-3% agarose gel; around 2-3% was made by diluting 2-3gr of agarose in 100ml of buffer TAE 1X at pH 8 that was heated up until boiling. After being heated up, it was left cool (around 60ºC) and added with DNA staining (1μL/30 ml). Agarose solution was poured into the agarose gel container, and the electrophoresis comb was installed at one of the agarose gel tips. After the gel is solid, the comb was removed carefully [19]**.** The container position was set and 500mL of buffer TAE 1X pH 8.0 was poured into the electrophoresis tank. Furthermore, 10μL of DNA sample mixture was poured into the agarose gel well; the voltage, electric current, and running time were set at 100V, 1A, and 100 minutes consecutively. After the electrophoresis finished, the gel was placed in the UV Transilluminator; then, the visualized DNA bands were absorbed.

DNA Visualization. The result of the study was collected by placing gel into the UV Transilluminator by removing the gel from its template. The gel was exposed to ultraviolet (UV) lights. The gel image that was formed under the UV light was documented. The DNA bonded with Diamond Nucleic Acid Dye will be fluorescent under the exposure of UV lights.

Result

The Result of DNA Quality and Quantity Measurement

The concentration and the purity of DNA can be determined by using a spectrophotometer. The result from the DNA concentration measurement is shown in Table 1.

Table 1. The Result of DNA Concentration Quality and Quantity test using a Spectrophotometer

Sample	DNA Concentration (μg/mL)	Absorbance at λ260	Absorbance at λ280	Ratio
N	4.16	0.432	0.154	3.748
P1	24.260	2.425	2.163	1.125
P2	23.88	2.388	2.133	1.121

Description: N = A mouse without any treatment
P1 = First AOM and DSS-induced mouse
P2 = Second AOM and DSS-induced mouse

The DNA Visualization Result for β-catenin gene

Based on the electrophoresis towards the β-catenin gene fragment collected using PCR with 5'-primer and 3'-primer, it was obtained several DNA bands, namely P1 of 346bp, 227bp, and 152 bp, P2 of 383bp, as well as N of 366bp and 341bp, shown in Figure 1. From the PCR products, cutting was performed using Hinf1 and EcoR1 restriction enzymes that resulted in N of 241bp, P1 of 241bp and 191bp, and P2 of 241bp, 137bp, and 89bp shown in Figure 2.

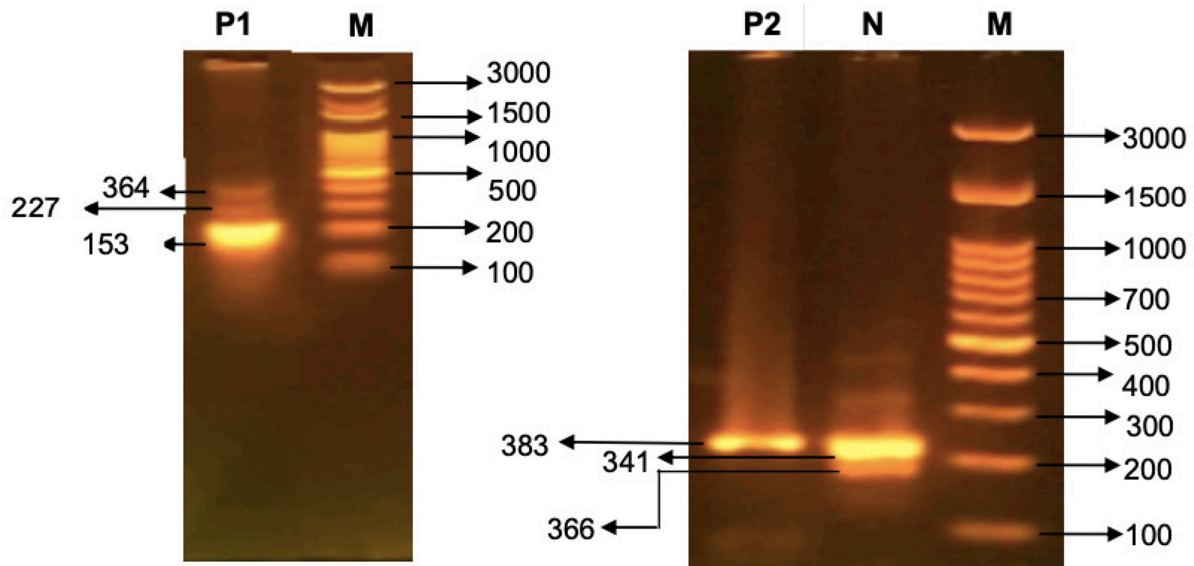

Fig 1. The Result for Digestion of PCR Products towards β-catenin Fragments using 5' prmer and 3'-primer

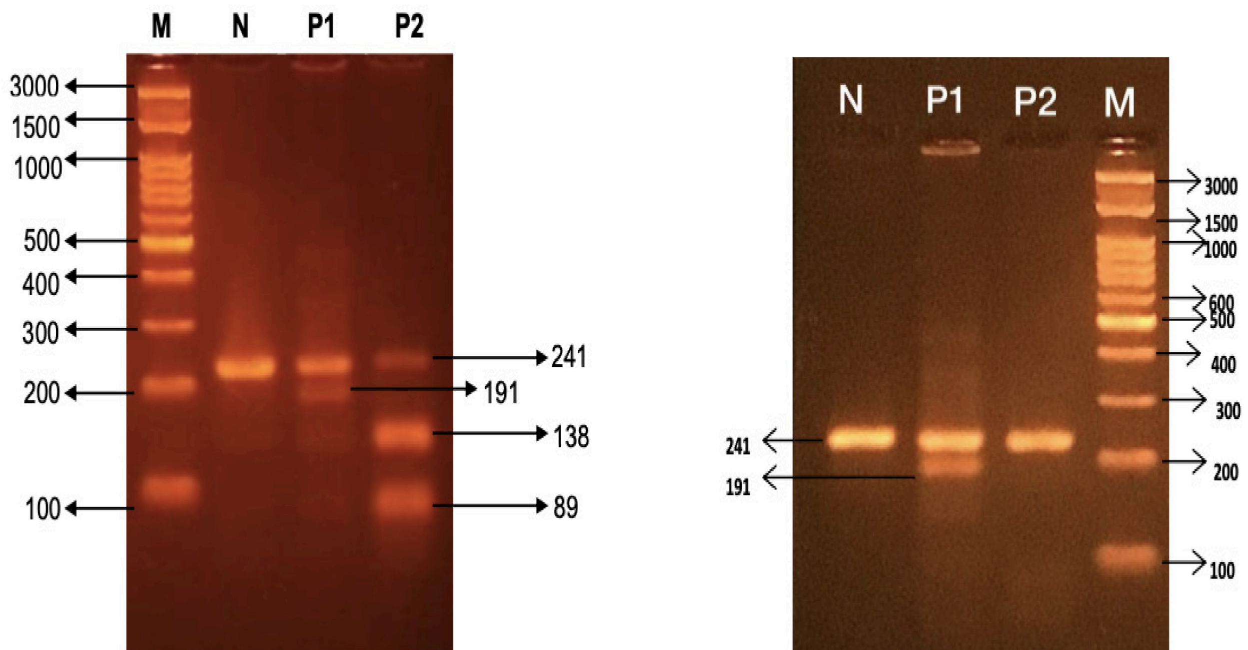

Fig 2a. The Result of Digestion of PCR Products for β-catenin gene fragments using Hinf1 restriction enzyme

Fig 2b. The Result of Digestion of PCR Products for β-catenin gene fragments using EcoR1 restriction enzyme.

Discussion

Based on the collected result against three different samples, N had a DNA concentration of 41.6 with a ratio of 3.784, indicating that it is contaminated by RNA, while P1 had a DNA concentration of 24.260 with a ratio of 1.125, and P2 had a DNA concentration of 23.88 with a ratio of 1.121, indicating that the samples were contaminated by protein[20] mentioned that the purity level of DNA was correlated with DNA quality. The wavelength used for measuring the concentration using a spectrophotometer was 260nm for DNA and 280nm for protein. DNA is claimed pure if it has a ratio of around 1.8 - 2.0 [21]. The ratio of < 1.8 shows that the extracted DNA is contaminated by protein while a ratio of > 2.0 shows that the DNA is contaminated by RNA. Nevertheless, the contamination does not interfere with the PCR process so that it can be used as a PCR template [22].

The extraction process was conducted by involving the chemical substances that could help the process of separating DNA from other cell components. The lysis solution was used for accelerating the destruction of the colon. Process of discharging DNA from both nucleus and mitochondria by extracting or lysis was done by homogenization and adding lysis buffer to prevent broken DNA, and adding proteinase K is aimed at purifying the DNA from the protein contaminant[23]. Thick and clustered DNA bands (not dispersed) show a high concentration and the total extracted DNA is in a complete condition, while the dispersed DNA bands show a binding between detached DNA molecules during the extraction process; therefore, the DNA genome is cut off into smaller parts[24].

β-catenin is a multifunctional protein involved in two different cellular processes, namely the cell adhesion process and signal transduction. β- catenin is a transcription cofactor at the Wingless (Wnt) signaling pathway and the target of Adenomatous Polyposis Coli (APC) gene products that are implicated in several ferocities. The obstructed β-catenin pathway may have important results in tumor growth and progressivity. APC gene mutation can cause β-catenin accumulation in the cytoplasm and cell nucleus. Besides mutation that occurred in APC, mutation in β-catenin or the changes in the Wnt signaling pathway can implicate the important step of carcinogenesis [25]

The loss or lack of β-catenin protein in the cell membrane will result in lacking intracellular adhesion and changing the tumor cells to an invasive phenotype; moreover, it can trigger the occurrence of epithelial to mesenchymal transition (EMT) program. Free β-catenin in the cytoplasm is the secondary bonded with the molecule complex of Adenomatous Polyposis Coli (APC), Glycogen synthase kinase-3 beta (GSK3ß)/ serine-threonine kinase, Axin as Adapter Molecule, and Casein Kinase-1 (CK1) that mediate the phosphorylation process and rapid degradation of β-catenin by the ubiquitin-proteasome system. The process is regulated by the Wnt signaling pathway that plays a role in cell adhesion, proliferation, differentiation, and the epithelial to mesenchymal transition (EMT) process. The activation of Wnt signaling increases β-catenin stability in avoiding the degradation process by deactivating GSK3β which results in β-catenin accumulation in the cytoplasm. Free β-catenin in the cytoplasm will then migrate into the cell nucleus and form a complex binding with T- cell factor/lymphoid enhancer factor (Tcf/Lef), a family of the transcription factor, and leads to an activation of target genes, such as cyclin-D1 and c-myc [26][27].

Based fig 1 on the study, for Digestion of PCR Products towards β-catenin Fragments using 5' prmer and 3'-primer. From two colon samples of mice model (*Mus musculus*) that had been induced by azoxymethane and Dextran Sulfate Sodium and one colon sample without any treatment, it was obtained a result with different DNA band size, namely N (negative) with a DNA band size of 341bp and 366bp, P1 of 346bp, 227bp, and 153bp, and P2 with a DNA band size of 383bp by using the PCR method. Detect the β-catenin gene mutation in codon position 32, 33, 34, and 35, it requires a PCR product with a DNA band size of 227bp It is in line with a statement [11].

The restriction test was conducted in PCR products to detect β-catenin gene mutation using Hinf1 and EcoR1 restriction enzymes consecutively in 4% agarose gel with the recognition sequence, namely GANTC and GAATTC. The restriction enzyme test in PCR products using EcoR1 obtained a result shown Figure 2b by the formation of DNA fragments in a negative sample of 241bp, P1 of 241bp, and 191bp, while P2 had a DNA fragment with a band size of 241bp. The collected result using the EcoR1 restriction enzyme showed that there was no β-catenin gene mutation characterized by no fragment with a size of 81bp and 146 bp. β- catenin gene mutation will yield fragments with a size of 81bp and 146bp It is contrary to a statement [11]. This shows that the β-catenin gene cannot experience any mutation in mice (*Mus musculus*) induced by azoxymethane and Dextran Sulfate Sodium for 8 weeks using the restriction enzyme test with EcoR1 enzyme, while those that experience mutation occurred in P2 using the restriction enzyme test with Hinif1 enzyme; it is shown in Figure 2a The result of RFLP using the Hinf1 restriction enzyme showed three bands with different sizes, namely 241bp, 89bp, and 138bp, and the DNA bands of 89bp and 138bp that show the occurrence of mutation. This mutation happens due to changes in nucleotides in codon position 32 or 33[11]. It indicates that the β-catenin gene experiences mutation in mice (*Mus musculus*) induced by azoxymethane and Dextran Sulfate Sodium for 8 weeks using the Hinf1 restriction enzyme test. Through this study, it can be seen that the occurrence of mutations in the β -catenin gene as a marker of colorectal cancer can be identified using the Hinif1 enzyme with the RFLP PCR method.

The limitation in this study is that the use of conventional PCR tools takes a long time to determine the optimal time according to the primer used. The weakness in this research method is that the results of the study have not been able to determine changes in the beta catenin sequence.

Conclusion

Based on the study that has been conducted, This study aims to identify the β-catenin gene encoding colorectal cancer in mice (Mus musculus) induced by azoxymethane (AOM) and Dextran Sulfate Sodium (DSS) using PCR RFLP (Restriction Fragment Length Polymorphism) method with enzyme EcoR1 and Hinf1, it can conclude that the result of RFLP against the PCR products using Hinf1 restriction enzyme shows mutation in P2 by the occurrence of band formation of 89bp and 138bp, while the testing of restriction using EcoRI shows no a mutation in the sample that is characterized by no DNA band formation with a size of 82bp and 146bp in the colon sample that has been induced by azoxymethane and Dextran Sulfate Sodium for 8 weeks. Further research is recommended to perform DNA sequencing to determine the type of DNA mutation of the β-catenin gene that occurs.

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